Structural Studies of Paramyosin. I. Hydrogen Ion Equilibria*

LYNN M. RIDDIFORD AND HAROLD A. SCHERAGA

From the Department of Chemistry, Cornell University, Ithaca, New York

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Hydrodynamic measurements on paramyosin (prepared from the adductor muscle of *Venus mercenaria*) indicated that the protein was homogeneous in various solvent media. The values of $s_{20,w}$ in 0.6 M KCl at pH 7.0 and in a denaturing solvent GU (5 M guanidine hydrochloride, 1.2 M urea, 0.3 M KCl) at pH 2.0 and 5.7 were similar and approximately 3.3 S. The molecular weight, determined by the Archibald approach-to-equilibrium method, depended upon the ionic strength and pH of the medium and varied from 228,000 to 618,000. In 0.3 M KCl at pH 7.4, the molecular weight, determined by the sedimentation equilibrium method, was 330,000. Lateral aggregation of the asymmetrical paramyosin molecule was proposed to account for the high molecular weights obtained. A comparison of direct and spectrophotometric titrations in 0.3 M KCl and in GU showed that nine tyrosyl groups are masked in native paramyosin and ionize normally in GU. The carboxyl and imidazole groups show normal ionization behavior in both 0.3 M KCl and GU. Since all the groups titrating in the alkaline range of the KCl solution (tyrosyl, lysyl, arginyl) have slightly low pK_{int} values which become normal in GU, local electrostatic interactions in KCl may be involved. An apparent absence of electrostatic effects was noted in alkaline GU solutions. Otherwise, the electrostatic interaction parameter, w, in both solvents agreed with the theoretical w based on a cylindrical model. It is concluded that the extreme stability of the native paramyosin molecule arises mainly from nonpolar interactions.

The ability of molluscan smooth adductor muscles to maintain tension over long periods has long been known (see Marceau, 1909, for an early description of the morphology, histology, and comparative physiology of these muscles). Schmitt *et al.* (1947) first applied the term paramyosin to the major protein component of these muscles, which is thought to be responsible for certain regular structural features (Bear, 1944; Hall *et al.*, 1945; Jakus *et al.*, 1944). When the protein was extracted from these muscles, it was called by several different names. Bailey (1956) and Kay (1958) called it "invertebrate tropomyosin," Kominz *et al.* (1957) called it "tropomyosin A," and Hodge (1952a, b) and Johnson *et al.* (1959) called it "paramyosin." Since a comparison of amino acid analyses and other properties (Szent-Györgyi, 1960) shows that these proteins are very similar, the name "paramyosin" will be used throughout this paper.

In the past few years, studies have been made of the mechanism of action of adductor muscles and the role that paramyosin plays in this mechanism. Some workers (Johnson *et al.*, 1959; Johnson and Philpott, 1959; Philpott *et al.*, 1960; Ruegg, 1959, 1961) favor the "catch mechanism," which can be explained on the basis of the crystallization properties of paramyosin (Johnson *et al.*, 1959). Others (Lowy and Millman, 1959a, b; Abbott and Lowy, 1960; Elliott and Lowy, 1961; Hanson and Lowy, 1961) favor a mechanism involving tetanic contraction in which the very slow rate of breakage of "tonic linkages" (those of filaments with paramyosin structure), *i.e.*, a sliding filament mechanism (Elliott and Lowy, 1961; Hanson and Lowy, 1961), is responsible for passive tension (Lowy and Millman, 1959a). However, Jewell (1959) and Prosser (1960) feel that these two hypotheses should be combined because actual physiologic action shows features of both mechanisms. Whichever hypothesis is most appropriate, the protein paramyosin is considered to be responsible for the phenomenon of sustained contraction.

Paramyosin has been found to be very stable in solution; *e.g.*, 9.5 M urea does not appear to denature it (Cohen and Szent-Györgyi, 1957). This is a surprising observation, since paramyosin is a long rodlike molecule (Hodge, 1952b; Kay and Bailey, 1959), mainly in the form of a stable α-helix (Cohen and Szent-Györgyi, 1957; Kay and Bailey, 1959). The question then arises of what type of tertiary structure (Scheraga, 1961) is responsible for the extreme stability of this protein molecule, since no disulfide bonds are present (Szent-Györgyi *et al.*, 1959). In an attempt to answer this question, studies of hydrogen ion equilibria of paramyosin were made.

Titration studies of paramyosin had been carried out previously by Johnson and Kahn (1959). However, a more detailed quantitative analysis of the titration data was necessary in order to obtain information about possible interactions between ionizable groups. Therefore, titration data were obtained on both the native protein and on a partially denatured one in which some of the abnormal

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ionization behavior could be eliminated.[1] Although *Venus* paramyosin does not appear to be completely denatured in 9.5 M urea at 20° (Cohen and Szent-Györgyi, 1957, 1958) or in 5 M guanidine sulfate (Harrap and Doty, 1960), it was hoped that the GU combination would be a better denaturant.

Experimental

Materials.—Paramyosin was extracted from the "white" portion of the adductor muscle of *Venus mercenaria,* obtained from Blount Seafood Co. (Providence, R. I.). Deionized water was used throughout the study; all chemicals used were reagent grade.

Eastman guanidine hydrochloride was purified according to the method of Kolthoff *et al.* (1957) and Mallinckrodt urea was recrystallized from absolute ethanol. Both reagents were dried at 50° *in vacuo* for 5 hours. A fresh solution of 8 M urea was found by the colorimetric method of Werner (1923) to show no trace of cyanate ion (Stark *et al.*, 1960).

Carbonate-free KOH containing 0.3 M KCl, prepared by the method of Kolthoff (1922), was standardized against potassium acid phthalate. HCl containing 0.3 M KCl was then standardized against the KOH.

Preparation of the Protein.—The preparative method of Johnson *et al.* (1959) was used with a few changes (Szent-Györgyi, 1959). The umbo portion of the shell adjacent to the dorsal margin was cracked, and pieces of the shell were chipped off in both anterior and posterior directions. The white portion of the two adductor muscles was separated from the pink-tinted portion. The excised muscle was placed in a beaker surrounded by ice water, and 10 volumes of cold 0.1 M KCl were added. The following steps were done at 4° except where noted. All centrifugation was done in a refrigerated Servall centrifuge at 0° at 3000 rpm except where noted. The suspension was blended for one minute in the Waring blendor and then centrifuged for 2 hours. The precipitate was washed three times with 20 volumes of cold 0.1 M KCl and centrifuged for 10 minutes after each washing. All supernatants were discarded. After extraction for 15 minutes with gentle stirring in three volumes of 0.6 M KCl, 0.04 M pH 7.5 Tris buffer, the extract was separated by filtration through cheesecloth[2] and the residue discarded. Three volumes of 95% ethanol were added to the extract at room temperature (25°) to denature the actomyosin. After 2 hours at room temperature, the precipitate was separated by a 30-minute centrifugation, resuspended in 0.6 M KCl, 0.01 M pH 7.0 phosphate buffer, and dialyzed for 24 hours against 6 volumes of the same solvent. Centrifugation (after dialysis) for 10 minutes at 10,000 rpm sufficed to remove the last traces of precipitated denatured actomyosin. The supernatant was then dialyzed against three volumes 0.01 M pH 6.0 phosphate buffer for 24 hours. The paramyosin crystals were recovered by 10-minute centrifugation at 10,000 rpm and subsequently redissolved in 0.6 M KCl, 0.01 M pH 7.0 phosphate buffer. For recrystallization of the protein, the pH 7.0 solution was dialyzed against 6 volumes 0.01 M pH 6.0 phosphate buffer for 30 hours. The crystals were treated as before except that, after they were redissolved, the solution was lyophilized. This procedure yielded a stable form of the protein, which was stored in the lyophilized state at 4°.

Solutions.—Protein solutions were prepared by dissolving the lyophilized sample in water with gentle stirring at room temperature for 2 to 3 hours. This solution was then dialyzed for 24 hours at 4° against the particular solvent to be used. After dialysis, solutions were clarified by centrifugation for 10 minutes at 4000 rpm at 0°. When the solvent GU[1] was used, a known volume of a 0.3 M KCl solution containing a known concentration of protein was added to weighed amounts of the recrystallized reagents.

The absorption spectrum of paramyosin was determined with a Beckman DU spectrophotometer. The spectrum showed a maximum at 277 mμ, a minimum at 250-253 mμ, and a value of 0.58 for the ratio of the optical densities at 260 and 280 mμ, indicating little, if any, nucleic acid contamination. Since these spectrophotometric characteristics were essentially the same as those obtained by Kominz *et al.* (1957), the extinction coefficient $E^{1\%}_{1cm}$ (277 mμ) = 3.05 (recalculated by Kominz, 1960, on the basis of 18.4% nitrogen content) was used to determine concentrations of the protein solutions.

Density and Viscosity Measurements.—The density of the GU solvent containing 0.3 M KCl was determined with use of a 5-ml pycnometer equilibrated in a constant-temperature bath maintained at (20.00 ± 0.05)°. Four determinations of the density of the GU solvent were made. To measure the viscosity of this same solvent, a 5-ml Ostwald type viscometer was used. The capillary tubing was approximately 13 cm long and 0.8 mm I.D. Outflow time for water at 20.0° was 46.8 ± 0.1 seconds, measured by a spring-driven stopwatch. At least ten replicate determinations of viscosity in a constant-temperature bath maintained at (20.00 ± 0.05)° were made.

Ultracentrifugation.—Measurements were made with the Spinco Model E analytical ultracentrifuge, equipped with an RTIC unit for temperature regulation within 0.1°. The sedimentation velocity and the Archibald approach-to-sedimentation-equilibrium experiments were performed at 20.0°. A sedimentation-equilibrium experiment was done at 4.0° for a period of 23 days. The conventional 12-mm cell was used with the 4° sector centerpiece. For acid solutions, the Kel-F centerpiece was used. Protein concentrations were generally between 0.5 and 0.6% except when serial dilutions were made.

[1] The intrinsic pK of acetic acid is affected oppositely by urea and guanidine; therefore, a mixed solvent (5 M guanidine HCl and 1.2 M urea, designated "GU") was used as a denaturing medium. The apparent pK of acetic acid is the same in GU as it is in 0.15 M KCl, and the apparent pK values of imidazole, phenol, and *n*-butylamine in GU differ only slightly from those in 0.15 M KCl (Donovan *et al.*, 1960).

[2] The cheesecloth had been boiled in 0.6 M KCl solution for 3 hours with several solution changes, then cooled in 0.6 M KCl to 4° prior to its use.

All measurements were made after enlarged patterns had been traced on graph paper.

Sedimentation velocity measurements were made at 59,780 rpm, and the sedimentation coefficient, s, was calculated according to the equation (Schachman, 1959):

$$s = \frac{2(x_2 - x_1)}{(x_2 + x_1)\omega^2(t_2 - t_1)} \quad (1)$$

where ω is the angular velocity in radians per second, t is the time in seconds, and x is the distance of the boundary in centimeters from the axis of rotation. The initial concentrations obtained by serial dilution were corrected for radial dilution during the experiment (Schachman, 1959). The time-dependent sedimentation coefficients were then plotted against concentration, and the line was extrapolated to infinite dilution by the method of least squares.

Molecular weight determinations by the Archibald approach-to-equilibrium method were conducted as outlined by Schachman (1957) and were performed at speeds from 4000 to 8000 rpm with the schlieren phase plate angles ranging from 70° to 85°. No silicone fluid was used. Consequently, the molecular weight was calculated only from the meniscus by the following equation (Schachman, 1957):

$$M = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \frac{\left(\frac{dc}{dx}\right)_m}{x_m c_m} \quad (2)$$

where R is the gas constant (8.314×10^7 ergs/mole/degree), T is the absolute temperature, $\bar{v}$ is the partial specific volume of the protein, and the subscript m denotes the position of the meniscus. The concentration at the meniscus, c_m, was computed by the equation (Klainer and Kegeles, 1955):

$$c_m = c_0 - \frac{1}{x_m^2} \int_{x_m}^{X} x^2 \left(\frac{dc}{dx}\right) dx \quad (3)$$

where X is an arbitrary x-coordinate in the plateau region $\left(\frac{dc}{dx} = 0\right)$, and where c_0, the initial concentration, is determined with a standard 12 mm, 4° sector synthetic boundary cell (Schachman, 1957). $\bar{v}$ is taken to be 0.728 at 20° C (an interpolated value from Kay's study [1960] of the effect of temperature on $\bar{v}$ of *Pinna nobilis* tropomyosin), and ρ is taken to be 1.028 for the 0.6 M KCl and 1.014 for the 0.3 M KCl solutions.

A speed of 1967 rpm was used for the attainment of sedimentation equilibrium of a 2-mm column of protein solution. Photographs were taken daily and analyzed. The results were plotted as $ln \left(\frac{1}{x}\right)\left(\frac{dc}{dx}\right)$ *vs.* x^2 (Schachman, 1957). When the data could be fitted by a straight line (*i.e.*, after the attainment of equilibrium), the method of least squares was applied. The molecular weight is the product of the slope of this line and $\frac{2RT}{(1 - \bar{v}\rho)}\omega^2$.

Direct Titration.—A Beckman pH meter (model GS, used on the "A" scale) was used for titrations in KCl and for determination of all pH values except those above pH 12. The TTTl type radiometer pH meter was used for pH determinations above 12 and for the titrations in GU. The pH meters were calibrated with either Beckman 4 and 7 buffers or 0.05 M potassium acid phthalate and equimolar phosphate buffers, prepared by the method of Bates (1954). In either case, 0.01 M borax (pH 9.18 at 25°), prepared by the method of Bates (1954), was used. For high pH measurements, standard Beckman buffer, pH 12.45, was also used. For the GS pH meter, a Beckman general-purpose glass electrode (39167) and a Ag-AgCl reference electrode containing 4 M KCl and an asbestos fiber junction (39168) were used. The GK 2021B probe type electrode was used with the TTTl radiometer.

The buret for delivering the titrant was a Gilmont ultramicroburet with glass plunger, of 1-ml capacity, graduated to 0.001 ml. The water-jacketed titration cell was specially designed to contain a volume of 5 ml yet allow a thick glass-enclosed stirring bar (magnetically driven from below) to be used without interfering with the electrodes. The electrodes and polyethylene capillary extensions of the two burets containing acid and base were inserted through a rubber stopper.

The water-jacketed cell was maintained at 25 ± 0.1° for the titration of the solvent blank and the protein solution. The pH meter was standardized both before and after the titration with approximately 5 ml of each buffer, and a maximum difference of 0.02 pH unit with the Beckman GS meter and of 0.01 pH unit with the radiometer was accepted. From a linear plot of the deviation between the actual and expected pH, each measured pH could be corrected. After 5 ml of the solution to be titrated was placed in the cell, the system was flushed with nitrogen, with stirring, for 30 minutes to allow for temperature equilibrium, removal of carbon dioxide, and denaturation (for the GU titrations). Then the titration was performed by the addition of successive increments of 1.002 N KOH (with the nitrogen still flowing) so as to change the pH about 0.2 unit each time, up to pH 12. Then 1.183 N HCl was added in the same manner down to about pH 1.7, followed by KOH back to pH 7 or to pH 12.

Readings were made only after the pH was stabilized (1 to 2 minutes). In the insoluble region, readings were made after longer periods of time (as much as 30 minutes).

For the KCl titrations, calculations were made with use of the apparent activity coefficient of hydrogen or hydroxyl ion calculated from the blank titration, according to the equations,

$$pH = -\log [H^+] - \log \gamma_H' \quad (4)$$

$$pK_w - pH = pOH = -\log [OH^-] - \log \gamma_{OH}'$$

where $[H^+]$ and $[OH^-]$ refer to the hydrogen ion and hydroxyl ion concentrations in solution, and γ_H' and γ_{OH}' refer to the apparent activity coefficients. These activity coefficients include any errors due to the glass electrode response and liquid junction potential (Tanford, 1950; Bates, 1954). It is assumed that the apparent activity coefficient is not affected by the presence of the protein. γ_H' was assumed constant from pH 3 to

pH 7, and γ_{OH}' was assumed constant from pH 7 to pH 11. When the values of r (moles of H^+ dissociated per mole protein) were calculated in this manner, correction for the change of volume due to the added titrant was made assuming additivity of the volumes. Since both the acid and base contained 0.3 M KCl, this assumption seems reasonable.

The calculation of the experimental titration curve (*i.e.*, values of r *vs.* pH) in GU was performed by subtracting the titration curve of the solvent from that of the protein solution. Blanks were titrated by increments of approximately 0.01 pH unit at the pH extremes. No volume correction was made. However, no error in protein concentration was introduced, since calculations were based on the total amount of protein present.

The molecular weight was taken to be 330,000, the value found in 0.3 M KCl at sedimentation equilibrium. The shape of the titration curve will be essentially unchanged if the molecular weight of the monomer is subsequently found to be different from 330,000. In this case, the values of r and Z will be changed by a common factor.

Spectrophotometric Titrations.—Spectrophotometric titrations in KCl were performed at various pH values between 7 and 13 either by adding amounts of 1.002 N KOH to the appropriately diluted protein solution or by diluting the protein solution with 0.01 M phosphate or borate buffer solutions (containing 0.3 M KCl). For the determinations above pH 13, the protein solution was added to known volumes of 1.002 N KOH. Appropriate volume corrections were made as in the direct titrations, assuming volume additivity. The optical density of the paramyosin solution at each pH was measured at 295 mμ against 0.3 M KCl at neutral pH.

For titrations in GU, a solution of known concentration of paramyosin in 0.3 M KCl was added to a concentrated GU solution to make the final concentration 5 M guanidine and 1.2 M urea. Known amounts of 1.002 N KOH were added to this solution and the appropriate volume corrections made. The optical density of the solution was measured at 295 mμ against GU solvent at pH 5. Since the optical density of GU is pH dependent, a solvent blank value was obtained in the same manner, and subtracted from the protein measurements. For both solvents the optical density measurements of the protein solutions were made at 5 minutes, 30 minutes, 1 hour, and 2 hours. For KCl solutions, measurements were made also after the solution was kept at 25° for 10 hours.

Results

Sedimentation Velocity.—The sedimentation velocity properties of paramyosin have been investigated in both KCl and GU solutions in order to compare the over-all size and shape of the native and the denatured molecule. The sedimentation patterns were also examined for evidence of any gross heterogeneity, but none was found (Fig. 1).

A plot of s_{20} *vs.* concentration in 0.6 M KCl, 0.01 M pH 7.0 phosphate buffer solution (Fig. 2) is seen to be linear over the concentration range examined. Extrapolation to infinite dilution by the method

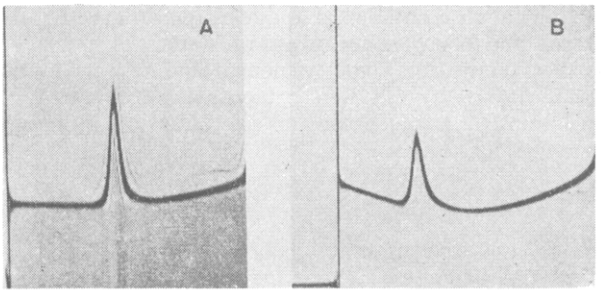

FIG. 1.—Typical sedimentation patterns of paramyosin at 20° at 59,780 rpm. *A*, in 0.6 M KCl 0.01 M phosphate buffer, pH 7.0, 4.5 mg/ml. *B*, in GU–0.3 M KCl, pH 5.7, 3.6 mg/ml.

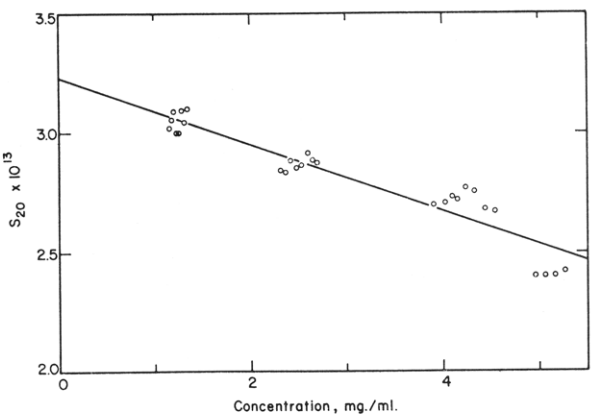

FIG. 2.—Sedimentation data for paramyosin in 0.6 M KCl, 0.01 M phosphate buffer, pH 7.0 at 20°, 59,780 rpm.

of least squares yields $s^\circ_{20} = 3.23$ S (S = 10^{-13} sec.). The same plot for unbuffered GU solutions at pH 2.0 and at pH 5.7 (Fig. 3) yields $s^\circ_{20} = (1.89 \pm 0.02)$S, indicating that the effective size and shape are independent of pH over this pH range in GU. In order to correct $s^\circ_{20,GU}$ to $s^\circ_{20,w}$, the experimentally determined values of 1.2825 centipoises and 1.1040 g/ml for the viscosity and density respectively of a GU–0.3 M KCl solution were used. The quantity $\bar{v}$ in GU solution was assumed to be about 1% less than $\bar{v}$ in aqueous salt solution as found by Kielley and Harrington (1960) for both myosin and ribonuclease in 5 M guanidine hydrochloride. This

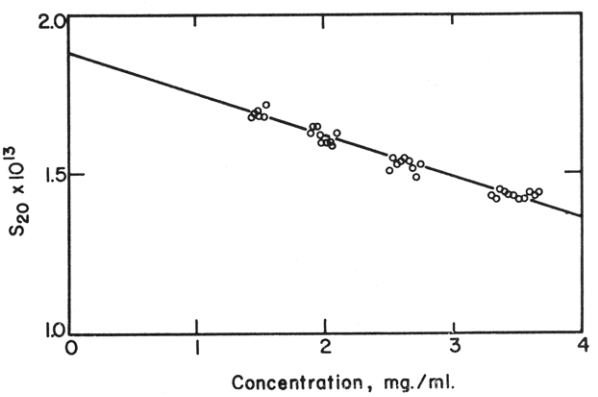

FIG. 3.—Sedimentation data for paramyosin in GU–0.3 M KCl, pH 5.7 at 20°, 59,780 rpm.

TABLE I

SEDIMENTATION COEFFICIENTS OF PARAMYOSIN FROM VARIOUS SOURCES

Source	Solvent	pH	$s_{20,w}$	Reference
Various invertebrates	0.2 M KCl	7–8[a]	*ca.* 3.0	Kominz *et al.*, 1957
Pinna nobilis	Phosphate-buffered KCl at μ = 0.6 and 1.1	7.0	3.1	Kay, 1958
Venus	0.033 M citrate	3.3–3.6	3.5	Hodge, 1952a
Venus	0.1 M KCl	3.3–3.6	4.3[b]	Hodge, 1952a, b
Venus	0.6 M KCl 0.01 M phosphate buffer	7.0	3.4	This work
Venus	GU–0.3 M KCl	2.0 and 5.7	3.2	This work

[a] Kominz, D. R. (1960). [b] Extrapolation to infinite dilution assuming the same concentration dependence as measured in 0.033 M citrate.

procedure may result in an erroneous value of $s_{20,w}$ if there is preferential interaction of the protein and the solvent (Schachman, 1959, pp. 228-236). The quantity $s^\circ_{20,w}$ was found to be 3.23 S for the GU solutions and 3.41 S for the 0.6 M KCl solution. These values of $s^\circ_{20,w}$ are compared with other values reported for paramyosin in Table I.

Molecular Weight.—A number of ultracentrifuge runs according to the technique of Archibald were made on solutions of varying ionic strength (0.3 to 1.3) and pH (6 to 8), and the results are shown in Table II.

TABLE II

MOLECULAR WEIGHT OF PARAMYOSIN FROM ARCHIBALD AND SEDIMENTATION EQUILIBRIUM RUNS

μ(Ionic strength)	pH	Protein Concentration (%)	Speeds	Molecular Weight (av.)[a]
1.3	6.1	0.34	7447–8225	618,000
0.6	7.0	0.73	5784–8225	228,000
0.6	7.8	0.54	4908–5784	257,000
0.3	7.8	*ca.* 0.6	4327–5563	350,000
0.3	7.4	0.74	2994	354,000
0.3	7.4	0.74	1967	330,000[b]

[a] Average of three different runs in each solution. (The standard deviation from the mean is about 6%, essentially owing to the error in the extrapolation of the dc/dx curve to the meniscus.) [b] Average of values from the final 4 days of the sedimentation equilibrium experiment.

On the 19th day of the sedimentation equilibrium experiment (0.3 M KCl, 0.01 M pH 7.4 phosphate buffer), the molecular weight approached a constant value; during the following 4 days, the molecular weight fluctuated about an average of 330,000 (mean deviation, 2%). Little evidence of aggregation was present (Fig. 4). A slight interaction (indicated by the curvature in Fig. 4) at the bottom of the cell was observed and can be explained by the absence of a silicone fluid layer (Ginsburg *et al.*, 1956). This layer was not used in order to avoid the silicone-protein interactions indicated in some preliminary runs by the Archibald technique.

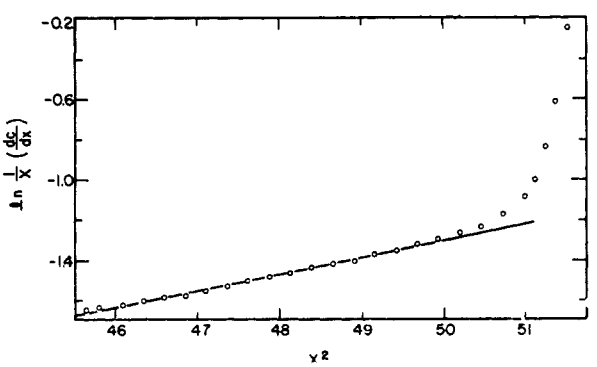

FIG. 4.—Typical sedimentation equilibrium data for paramyosin in 0.3 M KCl, 0.01 M phosphate buffer, pH 7.4, 20°, 1967 rpm. at equilibrium. This plot was made from data obtained on the 22nd day of the 23-day experiment.

Amino Acid Analysis.—The amino acid analysis of this preparation of *Venus* paramyosin was carried out by Analytica Corporation (118 E. 28th St., New York 16, N.Y.), and the results are presented in Table III. An analytical error of ±2% is

TABLE III

AMINO ACID COMPOSITION OF PARAMYOSIN[a]

Amino Acid	A Amino Acid Residue per 100 g Protein[b]	B Amino Acid Residue per 100 g Protein[c]
asp	13.44	14.73
thr	4.03	4.42
ser	4.06	4.45
glu	24.19	26.51
pro	0.27	0.30
gly	1.18	1.29
ala	8.00	8.76
val	3.64	3.99
met	1.95	2.14
ileu	3.54	3.88
leu	7.17	7.86
tyr	2.89	3.17
phe	1.03	1.13
lys	9.10	9.97
his	1.00	1.10
arg	13.64	14.95
try	0.40	0.44
CySH	0.39	0.42
Cysteic acid	0.64	0.70
½ cystine	0.47	0.51
amide NH_3	1.72	1.88
NH_3	2.25	2.46

[a] The present preparation analyzed by Analytica Corporation. [b] Assuming an average 100% recovery of amino acids from the protein. [c] Derived from average Kjeldahl nitrogen determinations on the basis of 18.4% nitrogen content (Kominz, 1960).

claimed by Analytica[3] after their application of the correction factors of 0.95 for methionine (column loss) and 0.92 for cysteic acid (hydrolysis loss). Further corrections for hydrolysis losses of 0.90 for serine and 0.95 for threonine and for the increase of ammonia corresponding to these two losses and to the ammonia content of HCl were made as outlined by Hirs (1960). The values were then converted from grams amino acid per 100 grams lyophilized sample to grams amino acid residue per

[3] A. S. Ostashever, Director, Biochemical Research Analytica Corporation, personal communication.

100 grams protein. The values in Column A in Table III were calculated on the assumption of an average 100% recovery of amino acids from the protein, and those in Column B were based on Kjeldahl nitrogen determinations made on the same sample (assuming an 18.4% nitrogen content, Kominz [1960]).[4]

In Table IV the values from Column A in Table III are converted to residues per mole protein, assuming a molecular weight of 330,000. These values are presented together with those of other preparations.

The amino acid values reported by the Analytica Corporation were obtained from ion-exchange chromatography and subsequent ninhydrin analysis of a 16-hour acid hydrolysate at 115° (Moore *et al.*, 1958; Spackman *et al.*, 1958). The tryptophan value was determined from a 3.5 N $Ba(OH)_2$ 10-hour hydrolysate at 100° which was subsequently chromatographed at pH 2.5 on a 15-cm column of Amberlite IR-120. The analysis of cysteic acid and methionine sulfone was done according to the procedure of Schram *et al.* (1954), and the sulfhydryl sulfur was determined as S-carboxymethylcysteine by the method of Cole *et al.* (1958). The total sulfur was determined by the colorimetric method of Vicera and Spevak (1956) and was found to be about 25% greater than the combined cysteic acid and methionine content. This colorimetric method is not as accurate as the micro-Carius gravimetric procedure, which could not be done owing to the very low sulfur content. Five times as much protein hydrolysate as usual had to be chromatographed for the S-carboxymethylcysteine analysis. Also, this amount of hydrolysate afforded a determination of the proline present, which was not detected in the usual chromatographic analyses. Total amide nitrogen was determined as described by Laki *et al.* (1954) and was found to be considerably less than the ammonia released during total hydrolysis.

Table V shows a comparison of certain typical characteristics calculated on the basis of values in Table IV. The only large discrepancy is noted in the percentage of nonpolar groups, but this can be attributed to the abnormally small leucine value for this preparation (Table IV), discussed below. The present preparation shows the lysyl:arginyl and glutamyl:aspartyl ratios which are characteristic of tropomyosin A (paramyosin) and distinguish it from tropomyosin B (Kominz *et al.*, 1957; Bailey and Ruegg, 1960).

Direct Titration.—The characteristic solubility behavior of paramyosin was noted during the titration in 0.3 M KCl (*i. e.*, an insolubility region between pH 3.5 and 6.5). With titration through a precipitate region, the question arises whether or not equilibrium between the protein and the hydrogen ion is achieved. The attainment of a stable pH with continuous stirring of the suspension after each addition of titrant was the experimental criterion of equilibrium. This criterion was shown to be quite adequate by the reproducibility and the reversibility of the titration through the entire pH range (Fig. 5). Thus, the paramyosin precipitate appears to allow free passage of hydrogen and hydroxyl ions, as suggested for the insulin precipitate in a similar

[4] The triplicate Kjeldahl nitrogen data from Analytica on this sample were not in precise agreement. Also, the total number of acid groups derived from Column A was 565 residues per mole protein (based on a molecular weight of 330,000), which fits the titration data in KCl and in GU, whereas the 623 residues per mole protein derived from Column B do not. Since no more sample remained for reanalysis, we are adopting the data in Column A rather than those in Column B. Although this introduces some uncertainty into our absolute values, it does not change our overall conclusions, as will be discussed below.

TABLE IV

AMINO ACID COMPOSITION OF *Venus* PARAMYOSIN AND *Pinna* TROPOMYOSIN PREPARED BY DIFFERENT PROCEDURES

	Residues/Mole Paramyosin[a]				
Amino Acid	*Venus*[b]	*Venus*[c]	*Venus*[d]	*Venus*[e]	*Pinna*[f]
asp	383	343	376	366	386
thr	131	122	119	119	78.6
ser	154	145	129	148.5	172
glu	614.5	521	558	558	601
pro	9.2	33.0	5.0	14.2	0
gly	68.0	85.8	49.5	69.3	42.9
ala	369	323	356	340	353
val	120	102	92.5	109	124
met	48.7	56.1	33.0	42.9	54.4
ileu	103	99.0	72.6	95.7	99.0
leu	208	310	350	340	360
tyr	58.2	69.3	59.4	89.1	49.5
phe	22.9	33.0	19.8		28.0
lys	233	218	195	205	224
his	24.0	25.1	? 13.2	? 19.8	14.8
arg	287	234	268	267	277
try	7.1	0	0	0	0
CySH	12.7				~6.8[g]
cysteic acid	14.0 15.0	~6.6-9.9	~6.6-9.9	~6.6-9.9	8.2[g]
amide N	331	350	363	363	383
NH_3	432.5				
Total groups	2859.6	2727.3	2704.0	2791.5	2872.4
% N	18.8[h]	18.1	18.4	18.1	18.9

[a] Calculated on the basis of a molecular weight of 330,000. [b] The preparation described in this paper and analyzed by Analytica Corporation. Derived from Column A, Table III. [c] Paramyosin prepared by A. G. Szent-Györgyi and analyzed by Kominz *et al.* (1957). [d] Paramyosin prepared by the method of Laki (1957) and analyzed by Kominz *et al.* (1957). [e] Kominz *et al.* (1957). [f] Bailey and Ruegg (1960). [g] Bailey (1957). [h] Calculated from the assumed 100% recovery of amino acids using the NH_3 value.

TABLE V

ANALYTICAL CHARACTERISTICS OF *Venus* PARAMYOSIN AND *Pinna* TROPOMYOSIN[a]

	Venus[b]	*Venus*[c]	*Venus*[d]	*Venus*[e]	*Pinna*[f]
Total free acid groups	565[g] (19.8%) 666.5[h] (23.4%)	514 (18.9%)	571 (21.2%)	561 (20.2%)	604 (21.1%)
arg + lys	520 (18.2%)	452 (16.6%)	463 (17.2%)	472 (17.0%)	501 (17.5%)
Net charge at pH 8	45[g] (1.6%)	62 (2.3%)	108 (4.0%)	89 (3.2%)	103 (3.6%)
Maximum + charge	544 (19.0%)	477 (17.5%)	476 (17.7%)	492 (17.7%)	516 (18.0%)
lys/arg	0.78	0.93	0.73	0.78	0.81
glu/asp	1.60	1.49	1.48	1.52	1.56
Average residue weight	115				115
Nonpolar groups	940 (32.9%)	1009 (37.0%)	973 (35.0%)	1017 (36.4%)	1061 (36.9%)
Amide N	11.6%	12.9%	13.5%	13.0%	11.0%
NH_3 N	15.2%				

[a] Calculated on the basis of a molecular weight of 330,000. [b] The preparation described in this paper and analyzed by Analytica Corporation. Derived from column A of Table III. [c] Paramyosin prepared by A. G. Szent-Györgyi and analyzed by Kominz *et al.* (1957). [d] Paramyosin prepared by the method of Laki (1957) and analyzed by Kominz *et al.* (1957). [e] Kominz *et al.* (1957). [f] Bailey and Ruegg (1960). [g] Corrected for ammonia N. [h] Corrected for amide N.

study by Tanford and Epstein (1954).

In GU the protein was soluble over the whole titration range of pH 1.7-12.0. Both reversibility and reproducibility are indicated in Figure 6.

between pH 5 and 6 (Szent-Györgyi, 1960). An attempt to find the isoionic point by desalting a paramyosin solution in 0.3 M KCl on a mixed bed resin proved to be inconclusive due to the insolu-

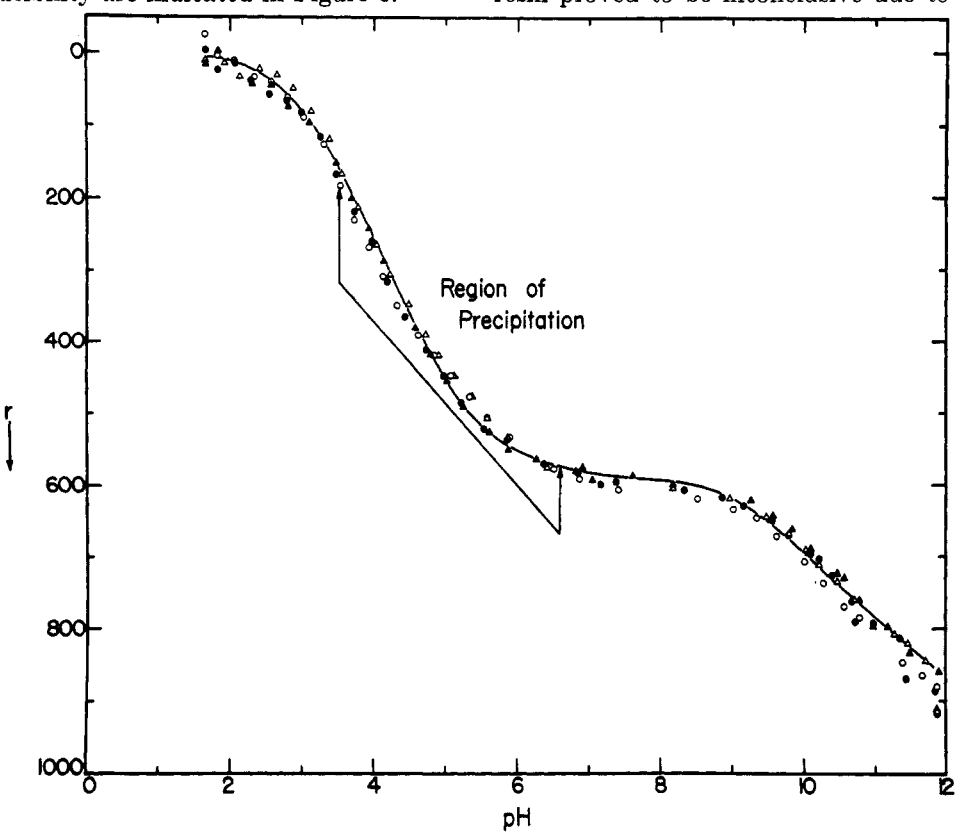

FIG. 5.—Titration curve of paramyosin in 0.3 M KCl at 25°. The circles and triangles denote two separate experiments, the open symbols representing titration with HCl and the filled ones representing titration with KOH. The curve is a theoretical one calculated from parameters in Table VI.

The theoretical curves seen in Figures 5 and 6 were computed as discussed below on the assumption of no ion binding (other than H^+) and on the basis of the parameters in Table VI. At low pH the curves are seen to flatten. The titration curve in 0.3 M KCl could be extrapolated to $r = 0$, where r is the number of protons dissociated per molecule, in order to obtain the maximum net positive charge, Z_{max}. However, to determine Z_{max}, the isoionic point also must be known. The isoelectric points of all the fibrous muscle proteins are known to lie

bility of the protein in this pH region. Consequently, several different isoionic points were arbitrarily chosen in the pH range 5.5 to 6.5, and the corresponding Z values were calculated from the experimental data in both solvents with 544[5] used for the maximum number of basic groups as indicated by the amino acid analysis[4] (Tables III and IV).

[5] If the maximum number of basic groups is 599 (derived from Column B, Table III), the excess number of acidic groups would be 24 instead of 22, which is not likely to alter the isoionic point significantly.

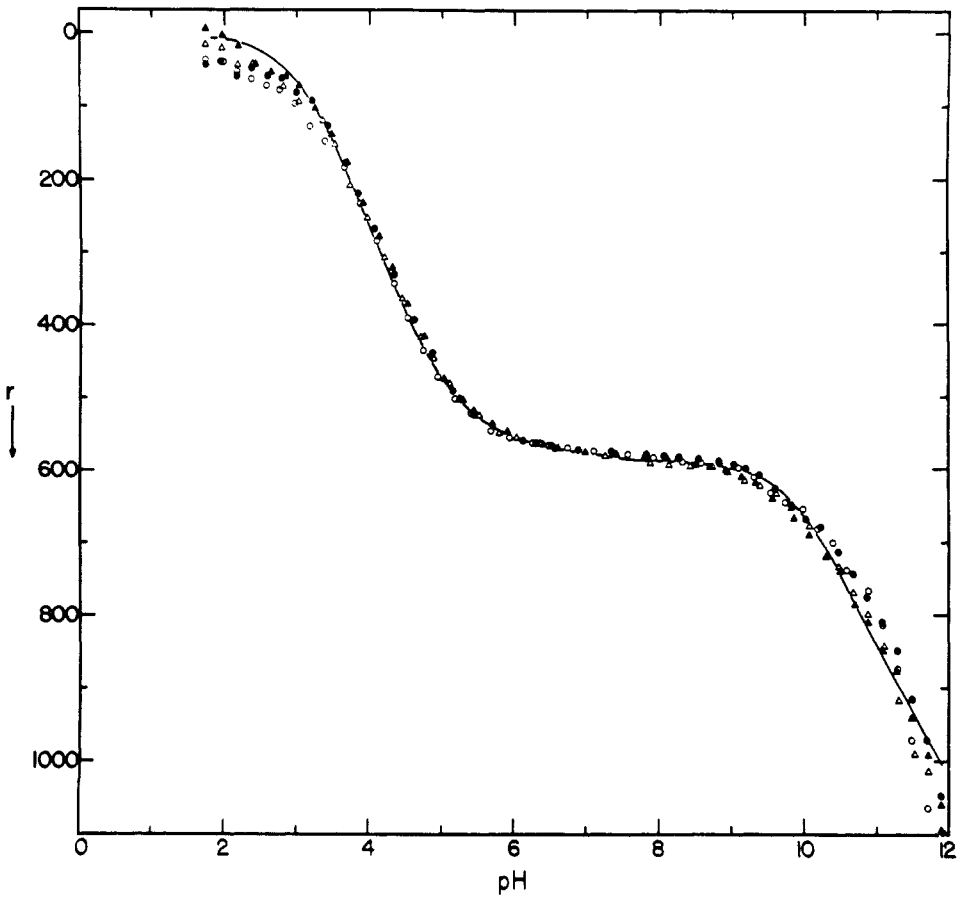

FIG. 6.—Titration curve of paramyosin in GU–0.3 M KCl at 25°. The symbols have the same meaning as in Figure 5.

Theoretical curves were calculated, and an isoionic point of approximately pH 5.8 was found to give the best fit of the theoretical curve to the experimental points in both solvents (Fig. 5 and 6). The scatter of experimental points from pH 2 to 3 in GU prevents an accurate extrapolation to $r = 0$, so the solutions aged as long as 10 hours at 25°. At pH levels above 13.2, the solutions became very milky after 2 hours. In GU there was a slight difference between the immediate measurement (within 3 minutes) and the 30-minute measurement, but no further difference after 1 and 2 hours.

TABLE VI
PARAMETERS USED TO CALCULATE TITRATION CURVES

	0.3 M KCl			GU-0.3 M KCl			
Group	Number	pK_{int}	w	Number	pK_{int}	w	ΔpK[a]
Carboxyl	565	4.55	1.91×10^{-3}	565	4.37	1.26×10^{-3}	−0.18
Imidazole	24	6.40	1.91	24	6.80	1.26	0.40
Sulfhydryl	14	9.50	5.21	14	10.00	6.34×10^{-5}	0.50
Tyrosyl	49	9.62	5.21	58	10.22	6.34	0.60
Lysyl	233	9.65	5.21	233	10.60	6.34	0.95
Arginyl	287	11.50	5.21	287	12.00	6.34	0.50

[a] $\Delta pK = pK_{GU} - pK_{KCl}$.

theoretical and experimental curves were adjusted to coincide in the region of pH 7 (where not many groups were titrating), the same number of carboxyl and imidazole groups being used as were found in 0.3 M KCl. The curves in the two solvents also coincide approximately in the neutral pH range.

Spectrophotometric Titration.—The spectrophotometric titration curves of the tyrosyl groups in 0.3 M KCl and in GU–0.3 M KCl at 25° are shown in Figures 7 and 8 respectively. Both reproducibility and reversibility are demonstrated. In 0.3 M KCl there was no apparent time dependence in

The change in the molar extinction coefficient, E, at 295 mμ in GU is 154,000 for 58 tyrosyl groups, or 2660 per group. Assuming this value of 2660 per group to be valid in 0.3 M KCl, as has been found for ribonuclease in the two solvents (compare Tanford *et al.*, 1955, and Cha and Scheraga, 1960), it was found that only 49 tyrosyl groups were apparently titrating in KCl.[6] About pH 13.7

[6] If there were 64 tyrosyl residues (as derived from Column B, Table III), the molar extinction coefficient would be 2410 per group. There would then be 54 groups apparently titrating in KCl, or 10 less than in GU. The difference between 9 and 10 buried tyrosyl groups is only 1.7%, which lies within experimental error.

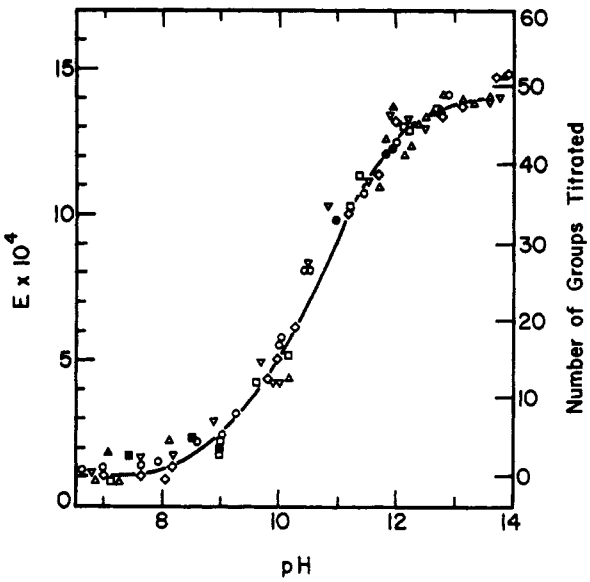

FIG. 7.—Spectrophotometric titration curve of paramyosin in 0.3 M KCl at 25° and at a wavelength of 295 mμ. The symbols of various types denote separate experiments. The closed symbols represent the reverse titration.

there is seen to be a slight indication that these groups are beginning to titrate (Fig. 7).

DISCUSSION

Hydrodynamic Properties.—No evidence of heterogeneity (Fig. 1) or aggregation is shown by paramyosin in a solution of given ionic strength. The linearity of the *s vs. c* plot (Fig. 2) and its negative slope give no indication of a reversibly aggregating system (Schachman, 1959). Furthermore, the molecular weight, determined by the Archibald method, at the meniscus did not decrease with time of centrifugation (Schachman, 1959).

However, the molecular weight of this preparation does indicate aggregate formation, since it appears to depend upon ionic strength and pH. The molecular weights (Archibald method) reported here (pH 7 to 8, μ = 0.3 and 0.6) are approximately integral multiples of the sedimentation-diffusion value of 131,000 (2× and 3×, within 10%) found by Kay (1958) for *Pinna nobilis* tropomyosin at pH 7, μ = 1.1. There also must be aggregation at acid pH, since, for *Venus* paramyosin in acid solutions (pH 3.3 - 3.6), Hodge (1952a) found a decrease in the sedimentation-diffusion molecular weight from 420,000 to 150,000 as ionic strength was decreased from 0.1.

This phenomenon of an increase in molecular weight with decreasing ionic strength at neutral pH, but a decrease in molecular weight with decreasing ionic strength at acid pH, suggests a correlation between solubility and molecular weight. The isoelectric point of this protein lies between pH 5 and 6 (Szent-Györgyi, 1960). Below this point, at pH 3.0 to 3.5, increasing the ionic strength to 0.3 causes precipitation (Hodge, 1952a). The crystallization point lies between pH 6.0 and 7.5 and depends upon ionic strength (Johnson *et al.*, 1959; Locker and Schmitt, 1957), but in an opposite manner (a decrease in ionic strength at a given pH causes crystallization). As the ionic strength is decreased, aggregation at a given pH (*e.g.*,7.0) may well occur before crystallization can be observed. This aggregation would manifest itself in the hydrodynamic properties, which would explain even the molecular weight of 618,000 (pH 6.1, μ = 1.3). The work of Locker and Schmitt (1957) can be cited as evidence in support of this viewpoint. They found that addition of four parts of water to a 0.6 M KCl, phosphate-buffered solution at pH 7.5 caused a flocculent amorphous precipitate which crystallized upon standing. Aggregation was also observed in preliminary electron microscope studies of the present preparation by the spray technique (Brill and Siegel, 1961). However, a systematic study of the molecular weight as a function of ionic strength, pH, and concentration is necessary to resolve this question.

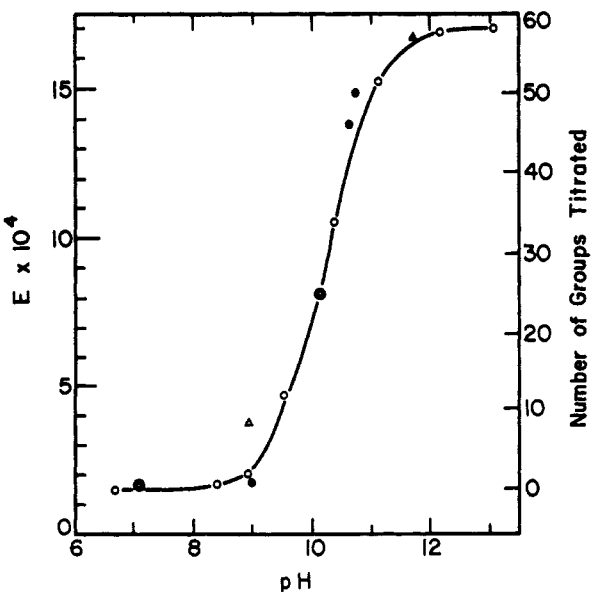

FIG. 8.—Spectrophotometric titration curve of paramyosin in GU–0.3 M KCl at 25° and at a wavelength of 295 mμ. The symbols have the same meaning as in Figure 7.

In spite of the difference in molecular weights found by Kay (1958) and Hodge (1952b), both workers determined the length of the molecule tc be about 1400 A and concluded that the molecule was rodlike. Hodge (1925a) also found a progressive decrease in apparent molecular diameter as the molecular weight decreased. Therefore, aggregation seems to occur side by side rather than end to end. All the observed periodicities of paramyosin "crystals" obtained by reducing the ionic strength of a 0.6 M KCl solution at neutral pH (Locker and Schmitt, 1957; Hodge, 1959) can be related to the 1400 A length by arrangements involving axial displacements (Hodge, 1959). Our preparation is presumably a lateral aggregate and may involve some type of supercoiled α-helices as postulated by Bear and Selby (1956). A two-chain coiled coil conformation has been found to give excellent agreement with hydrodynamic data (Cohen, 1961).

The similarity of the $s_{20,w}$ in KCl and in GU at

room temperature (Table I) may be indicative of either a similar size and shape of the paramyosin molecule in the two solvents or a compensating change of molecular weight and partial specific volume with the frictional coefficient. Since the molecule retains one third of its helical structure in GU at room temperature (Riddiford and Scheraga, 1962) and probably consists of random regions interspersed with helical regions, a similar size and shape may be maintained by intermolecular interactions involved in the coiled coil conformation (Cohen, 1961). However, both the size and shape may change in GU. No molecular weight studies have been made in GU, since the theory has not been adequately worked out for multicomponent systems (Schachman, 1959, pp. 228–236). Even the value of $s_{20,w}$ for the GU solution is at best approximate.

If precise data were available, the calculation of the hydrodynamic parameter $\beta(p)$, which is characteristic of the size and shape of the effective hydrodynamic ellipsoid (Scheraga and Mandelkern, 1953), would distinguish between the various possible forms. The one essential criterion is that for a given solvent condition, only a single species exist; and this appears to be true. A systematic study of molecular weight, sedimentation coefficient, and intrinsic viscosity of the same preparation under identical solvent conditions (temperature, ionic strength, and pH) is needed.

Amino Acid Analysis.—The comparison of the amino acid content of our preparation of *Venus* paramyosin with the reported values for *Venus* (Kominz *et al.*, 1957) and *Pinna* (Bailey and Ruegg, 1960) tropomyosins (Table IV) shows favorable agreement in most instances. The large discrepancy in the leucine value may be a result of some experimental error and casts doubt upon the assumption of an average 100% recovery of amino acids from the protein. However, as pointed out previously,[4] values derived when this assumption is used fit the experimental titration data better, so are used in spite of this discrepancy in the leucine value.

The presence of a small amount of tryptophan (0.25%) may indicate a slight impurity, since all other preparations of paramyosin have a negligible amount (Kominz *et al.*, 1957; Bailey and Ruegg, 1960; Bailey, 1957). Perhaps the impurity also accounts for the proline value of $2.8/10^5$ g, which is somewhat higher than the value of $1.7/10^5$ g which Kominz *et al.* (1957) considered to be characteristic of all tropomyosin A (paramyosin).

A comparison of the S-carboxymethylcysteine and the two values for cysteic acid indicates one possible disulfide bond in our preparation. Since the two reported cysteic acid values differ by one group, we can only conclude that there may be at the most one disulfide crosslink, and there may not be any, as reported by Szent-Györgi *et al.* (1959).

In Table V the total number of free acid groups for the present preparation was calculated on the basis of the ammonia value for the column. If the amide nitrogen value were used, the total number of free carboxyl groups would be much greater than the number found by titration (Table VI) and greater than the values given in the literature for both *Venus* and *Pinna* tropomyosins (Table V). For this reason we have placed more credence in the amino acid composition deduced from the ammonia value for the column.

Titrations.—Since the hydrogen ion equilibria in both solvents are seen to be reversible, the titration data may be interpreted according to the equation (Tanford, 1950):

$$\mathrm{pH} - \log \frac{x_i}{1 - x_i} = (\mathrm{pK_{int}})_i - 0.868\, wZ \qquad (5)$$

for each type of ionizable group, assuming their intrinsic identity. x_i represents the dissociated fraction of the groups of the ith kind at the given pH and the $(\mathrm{pK_{int}})_i$ is the negative log of the intrinsic dissociation constant at the ionic strength used. The term $0.868\, wZ$ corrects for any electrostatic interaction between the proton and the protein molecule of net charge Z. w is an empirical parameter which depends on molecular size, shape, and permeability at constant temperature and ionic strength. The ordinate of the theoretical titration curve is

$$r = \Sigma n_i x_i \qquad (6)$$

where n_i is the number of groups of the ith type.

The experimental parameter, w, can also be derived theoretically from the electrostatic free energy for an asymmetric rodlike macromolecule on the basis of a model for a cylinder with charges smeared over the surface (Hill, 1955). From this model,

$$w = \frac{Ne^2}{DRTl}\left[\frac{K_0(\kappa a)}{\kappa a K_1(\kappa a)} + \ln \frac{a}{b}\right] \qquad (7)$$

where N is Avogadro's number, e is the electronic charge, D is the dielectric constant, R is the gas constant, T is the absolute temperature, l is the length of the molecule, K_0 (κa) and K_1 (κa) are modified Bessel functions of the second kind, κ is the Debye-Hückel parameter, which depends upon ionic strength, b is the radius of the cylinder, and $a = b + 1$ in Angstroms (Hill, 1955) and is the radius of exclusion.

The data for the carboxyl and tyrosyl[7] groups in both solvents were plotted according to equation (5) and are shown in Figures 9 through 12. The values of Z were obtained from Figures 5 and 6, taking the isoionic point to be pH 5.8 and assuming no ion binding (other than H^+). The resulting points are seen to lie on straight lines within experimental error except for the plot of the carboxyl groups in GU (Fig. 11), which shows a decided change of slope at Z greater than 450, corresponding to pH values below pH 3.0. A similar phenomenon

[7] The value of x for the tyrosyl groups was obtained from the equation

$$x = \frac{E - 10{,}000}{140{,}000}$$

for the KCl titration (Fig. 7) and from an analogous equation

$$x = \frac{E - 15{,}500}{170{,}000}$$

for the GU titration (Fig. 8).

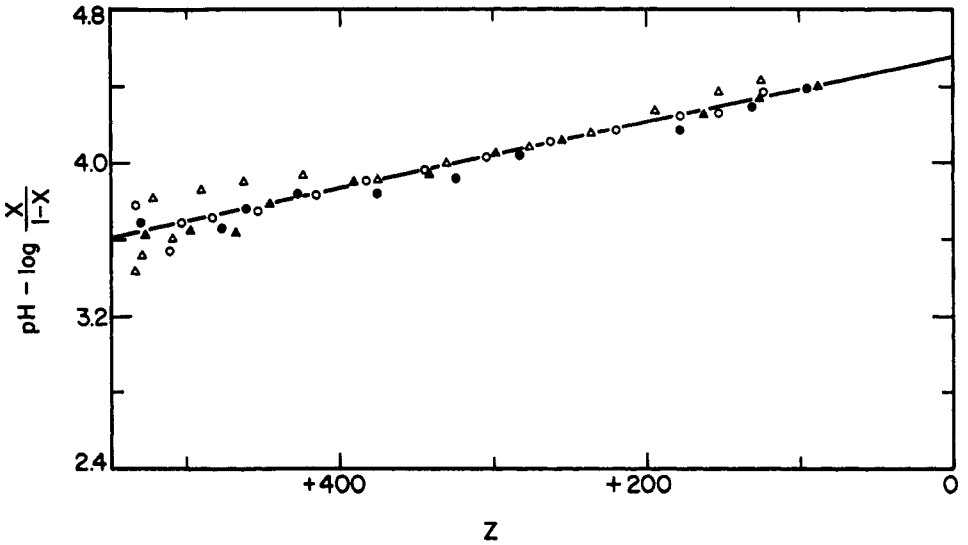

FIG. 9.—Plot of data for carboxyl groups in 0.3 M KCl according to equation (5). The circles and triangles refer to the same data as those in Figure 5. The line was computed by the method of least squares.

was observed for lysozyme in GU (Donovan *et al.*, 1960). When GU solutions at pH 2.0 and 5.7 were compared at 25°, there was no change in sedimentation coefficient or in optical rotatory properties (Riddiford and Scheraga, 1962). Therefore, the points below pH 3.0 were disregraded in determining the w and pK_{int} values from the slope and intercept at $Z = 0$.

The parameters determined from these plots are listed in Table VI, together with the pK_{int} values for the imidazole, sulfhydryl, lysyl, and arginyl groups; these were determined by a trial-and-error procedure to give the best fit to the experimental points. When the apparent changes in pK_{int} were compared to the changes noted for model compounds in transfer from 0.15 M KCl to GU (Donovan *et al.*, 1960), it was found that the difference was in the same direction except for the lysyl groups. Here there was a large increase (0.95) in pK_{int}, as compared to a decrease of 0.05 for *n*-butylamine. The particular G:U ratio used (1.2 M urea, 5 M guanidine hydrochloride) in the testing of model compounds was chosen so that the apparent pK of acetic acid was the same as in 0.15 M KCl (Donovan *et al.*, 1960). Owing to the solubility properties of paramyosin, 0.3 M KCl was used in the present experiments (with GU as well); but this difference in molarity cannot explain the discrepancy (see the plot of pK values for model compounds *vs.* molarity in Donovan *et al.*, 1959).

Since the numbers of the groups in Table VI will be multiplied by a common factor if their absolute values are wrong,[4] the shape of the titration curve will be essentially unchanged. Calculations, based on the data of column B of Table III, showed that the pK_{int} values and the w factors were not significantly changed from those calculated on the basis of column A of Table III.

All the groups that can be titrated in the alkaline region in 0.3 M KCl have slightly low pK_{int} values compared to other proteins (see Table VIII, p. 534, in Edsall and Wyman, 1958). In GU these pK_{int} values are within the normal range. Thus, it appears that the tyrosyl, lysyl, and arginyl groups are involved in some type of interactions in the native molecule which are destroyed by GU. One possi-

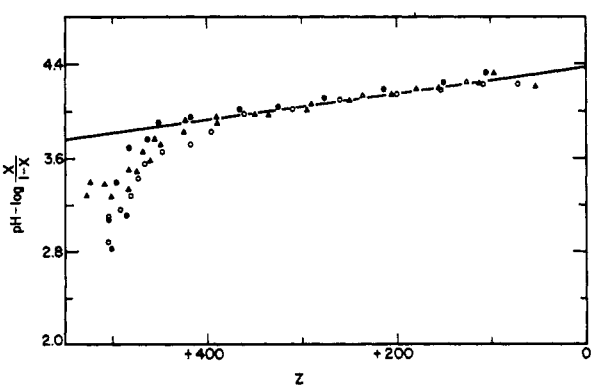

FIG. 10.—Plot of data for carboxyl groups in GU–0.3 M KCl according to equation (5). The circles and triangles refer to the same data as those in Figure 6. The line was computed by the method of least squares, disregarding all points with Z greater than 450 (*i.e.*, pH less than 3.0).

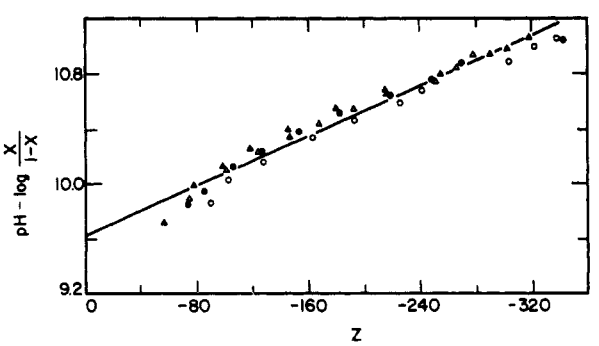

FIG. 11.—Plot of data for tyrosyl groups in 0.3 M KCl according to equation (5). The circles and triangles refer to the same data as those in Figure 5. The line was computed by the method of least squares.

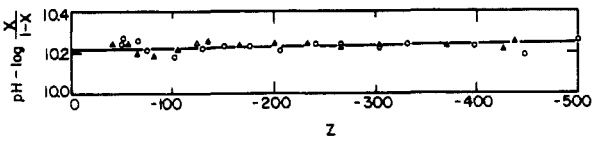

FIG. 12.—Plot of data for tyrosyl groups in GU–0.3 M KCl according to equation (5). The symbols refer to the same data as those in Figure 6. The line was computed by the method of least squares.

ble interaction is that of K^+ binding, since this would decrease the effective negative charge and thereby the pH at which a given degree of ionization is attained (equation 5). Its absence in GU–0.3 M KCl may be explained if the K^+ binding also involved hydrogen bonding, since GU causes the rupture of internal hydrogen bonds. In the case of myosin, a similar molecule, K^+ binding has been demonstrated (Nanninga, 1957; Lewis and Saroff, 1957), and interpreted in terms of a chelate formation (Saroff, 1957).

The noted inaccessibility of nine tyrosyl groups[6] in the native molecule with a pK_{obs} greater than thirteen cannot be explained solely by electrostatic interactions. Since the difference spectrum between a pH 2.8 and a pH 1.2 solution of *Venus* paramyosin shows peaks characteristic of tyrosyl hydrogen bonding (Harrap and Doty, 1960) (see also Laskowski et al., 1956; Scheraga, 1957), hydrogen bonding (Laskowski and Scheraga, 1954) may explain this inaccessibility, although all 565 carboxyl groups appear to titrate normally with a pK of 4.55. However, experimental error could easily compensate for the abnormal behavior of nine carboxyl groups (1.6% of the total number) because of the insolubility region between pH 3.5 and 6.5. The peaks in the difference spectrum are intensified upon heating (Harrap and Doty, 1960). To explain this phenomenon, Hermans and Scheraga (1961) have postulated that tyrosyl-carboxylate hydrogen bonds are embedded in a hydrophobic region in ribonuclease which is disrupted by heat, but not by acid. It is possible that the inaccessible tyrosyl groups may be in hydrophobic regions in the vicinity of the carboxyl groups, but not actually forming hydrogen bonds with them. Thus, the titration data reported here and the difference spectrum observed by Harrap and Doty (1960) could be reconciled.

The electrostatic correction term involves w, which appears to be pH dependent in both solvents to the extent that it differs at alkaline and acid pH. Since w should be regarded only as an empirical parameter (Tanford, 1955a), the experimental deviations noted below should be considered only as indications of a change in shape. The experimental w values from the carboxyl ionizations in both solvents (Fig. 9 and 10) are less than the theoretical value of 2.74×10^{-3} calculated on the basis of the dimensions (1400 A length, 9.3 A radius) found by Kay (1958). On the basis of equation (7) for a cylindrical model, a decrease of w of the order of magnitude noted is predicted if b is doubled or tripled (l and κ remain constant).

In alkaline KCl (Fig. 11) w increased somewhat, but in alkaline GU (Fig. 12) w decreased by two orders of magnitude. Two explanations may be advanced for this apparent absence of electrostatic effects. Either GU may have penetrated the molecule, causing a decrease in w analogous to the effect calculated by Tanford (1955b) for globular protein ions, or the net charge could be greatly different from the charge affecting the tyrosyl groups, as seems to be the case for lysozyme in GU (Donovan *et al.*, 1960). In order to normalize the nine "buried" tyrosyl groups, GU must have disrupted some interactions in the molecule without disrupting the whole structure (approximately one third of the helical structure is retained at 25° [Riddiford and Scheraga, 1962]). Subsequently, there may have been an opening or expansion of these parts of the molecule. Therefore, the difference in electrostatic behavior could be a result of the solvent surrounding the now accessible tyrosyl groups.

Johnson and Kahn (1959) found that 130 out of 800 amino acid residues of *Venus* paramyosin (based on 100,000 molecular weight) titrated with an observed pK of 6.3 in 0.3 M KCl and that there were apparently no carboxyl groups with a pK of about 4.3. Our results disagree with these findings. All 565 carboxyl groups titrate with a normal pK_{int} of 4.55 and all 24 imidazole groups titrate with a normal pK_{int} of 6.40, thus accounting for all the groups titrating in the pK range 6.0-6.5. The reason for the discrepancy is not clear, but could be concerned with the problems of titrating a protein through its insoluble range.

The hydrodynamic properties of *Venus* paramyosin are consistent with models of stable asymmetric rodlike aggregates. Even in GU, the rodlike character of paramyosin is apparently retained. Since hydrogen bonds have not been detected by titration—with the possible exception of nine tyrosyl interactions—electrostatic and hydrophobic interactions must be responsible for most of the stabilization of the native molecule (possibly a coiled coil). The stabilization of the partially unfolded molecule in GU at room temperature will be discussed in the following paper (Riddiford and Scheraga, 1962).

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